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Biopreservative Effect of Lactoferrin Against Foodborne Pathogens Inoculated in Egyptian Soft Cheese "Karish Cheese"

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ABSTRACT

Lactoferrin is a natural component that is used as a food additive to inhibit the growth of pathogenic microorganisms. This study was designed to evaluate the antibacterial effect of lactoferrin against *E. coli* O157:H7, *S. aureus L. monocytogenes*, and *B. cereus* inoculated in one of the most popular Egyptian soft cheese "Karish cheese". The sensory properties including appearance, odor and flavor of the cheese, to which lactoferrin was added, were evaluated. The results of sensory evaluation revealed that the samples treated with the different concentrations of lactoferrin were generally acceptable and did not negatively affected its sensorial properties. Moreover, the samples treated with high concentration of lactoferrin were given high score. Antibacterial effect of 1%, 2% and 4% concentrations of bovine lactoferrin against the foodborne pathogens were tested in vitro using agar well diffusion test to determine the appropriate concentration to be applied in cheese. The results showed that lactoferrin 4% concentration was the most effective concentration against all the examined foodborne pathogens, *E. coli* O157:H7, *S. aureus L. monocytogenes*, and *B. cereus*. The antibacterial effect of 4% lactoferrin against the before mentioned foodborne pathogens in Karish cheese was investigated and it could inhibit them during the cold storage period. This study highlighted that lactoferrin improved sensory properties of Karish cheese experimentally inoculated with some food borne pathogens such as *E. coli* O157:H7, *S. aureus L. monocytogenes*, and *B. cereus*, in addition to extending its shelf life at cold storage. Therefore, the addition of lactoferrin to Karish cheese could improve its microbiological safety.

1. INTRODUCTION

Cheese is popular and its consumption is increasing in many countries due to its health benefits, as it is rich in proteins, dietary calcium and phosphorus and addition to its characteristic flavor (CDIC, 2017, USDA/ARS, 2019).

Karish cheese is one of the most popular, oldest cheese varieties in Egypt, it is acid-coagulated fresh raw milk soft cheese, and accounts for around 50% of white soft cheese produced in Egypt (Ombarak et al., 2016).

Contamination of cheeses with foodborne pathogens such as *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Bacillus cereus* and *Listeria monocytogenes* is mainly because of the use of raw milk, or faulty processing, mainly during pasteurization or post-processing handling (Kousta et al., 2010). Foodborne illnesses linked to cheese consumption have occurred in many countries (CDC, 2010; CDC, 2018).

E. coli O157:H7 is an enterohemorrhagic bacterium that produces Shiga toxins, and causes various illnesses such as hemorrhagic colitis, diarrhea

and hemolytic-uremic syndrome (Kaper et al., 2004). Cheese-related *E. coli* O157:H7 outbreaks have been reported (CDC, 2010).

S. aureus is a ubiquitous pathogen that can cause staphylococcal food poisoning and various human infections (Le Loir et al., 2003). *S. aureus* contaminated cheese had caused many foodborne outbreaks as reported by Johnson (2001) in England, Simeão do Carmo et al. (2002) in Brazil and Ostyn et al. (2010) in France.

B. cereus is a spore forming bacterium that produces diarrheal and emetic toxins and causes foodborne illness (Sutherland et al. 1994).

L. monocytogenes is widely spread in the environment and causes the foodborne illness listeriosis (Jalali and Abedi 2008). However, the reported frequency of *L. monocytogenes* cheese-related outbreaks is low, the mortality rate is higher than that of other bacteria causing foodborne illnesses (Makino et al., 2005; Bille et al., 2006, FDA, 2012,). Fretz et al. (2010) reported that *L. monocytogenes* caused four deaths out of 14 cases in Austria and Germany in 2009 due to contaminated Quargel (acid curd cheese). Multistate outbreaks of listeriosis occurred in the USA between October 2008 and March 2009 due to Mexican style cheese (Jackson et al., 2011), and in 2014 due to cheese products (CDC, 2014). Even in a relatively low cheese consumption country like Japan, *L. monocytogenes* outbreaks were reported due to cheese consumption resulting in 86 cases of *L. monocytogenes* infection (Makino et al., 2005).

A promising class of antimicrobial compounds are lactoferrin (LF) and its peptides which is valuable in the fight against foodborne pathogens. LF belongs to the transferrin family, iron-binding proteins (Lonnerdal and Iyer 1995).

The bactericidal activity of LF is partly the result of the direct interaction between the positive charged regions with anionic molecules present on the surface of some microorganisms, resulting in increased membrane permeability, that lead to bacterial damage (Haversen et al. 2010). Moreover, LF possesses anti-fungal, antiparasitic, anti-viral, and anti-tumor properties. It endorses bone growth, protects the intestinal epithelium, and stimulates the retrieval of immune system functions. LF is involved in the treatment of hepatitis-C. It also improves the health status of Alzheimer's disease patients (Hao et al., 2019).

Soft cheese or cheese made from raw or unpasteurized milk have been generally linked to cheese related foodborne illnesses. Therefore, objective of this study was to assess antibacterial effect of lactoferrin on *Escherichia coli O157:H7*, *Staphylococcus aureus, Bacillus cereus and Listeria monocytogenes* added to laboratory manufactured Karish cheese, with the aim of evaluating its antibacterial effect to inhibit the growth of these pathogenic microorganisms.

2. MATERIAL AND METHODS

2.1. Bacterial strains and growth media:

*E. coli O157:H7 (*ATCC® 700728), *L. monocytogenes* (ATCC® 35152), *S. aureus* (ATCC® 43300) and *B. cereus* (ATCC® 10876) were obtained from Cairo-MIRCEN (Microbiological Resource Center), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. Before each experiments the strains were cultured on its selective media and maintained in Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA). To obtain a concentration of 10^4-10^5cfu/ml, each strain was inoculated in TSB and incubated at 37°C for 16 h. Thereafter, the culture suspensions were collected after centrifugation for 2 min at 3000 rpm. Pellets were washed and re-suspended in 10 ml of 0.1% sterile peptone water. Final count was confirmed by enumeration on TSA (Chen et al., 2007).

2.2 Lactoferrin

Bovine Lactoferrin 20,000 IU/mg (Sigma-Aldrich., U.S.A.) was used in this study. The following concentrations of LF solution were prepared, 1, 2 and 4% in distilled water and sterilized by 0.45 mm filter and freshly used.

2.3 Manufacturing of Karish cheese

Karish cheese was prepared as described by El-Khawas and Hassan (2015). Briefly, cow's milk was pasteurized at 80 ºC for 15 seconds, thereafter, cooled to 40 ºC then inoculated with rennet, 3g /100 kg, (Hansen Laboratories, Copenhagen, Denmark). All treatments were incubated at 40 ºC up to curding. Salt at 1% was added between cheese layers and the curd was left overnight to whey drain into small cheese molds at room temperature then the curd was divided into equal parts 200 gm each, then were directly packed into polyethylene packages and stored at 5°C for further treatment and addition of LF.

2.4 Sensory characteristics evaluation and determination of titratable acidity % (TA%)

The cheese samples sensory characteristics and acidity were examined at zero, 5^{th}, 10^{th}, 15^{th}, and 20^{th}

day of storage till appearance of spoilage signs. The sensory characteristics for both control and LF treated Karish cheese samples were evaluated according to International Dairy Federation recommendation (IDF, 1995; Clark, 2009). Briefly, Karish cheese samples were subjected to evaluation by staff members at the Food Hygiene laboratory, Faculty of Veterinary Medicine, Menoufia University. Evaluators were told to grade each sample according a weighted scale totaling 100 points: 20 points given for the appearance and color, 35 for body and texture and 45 for flavor.

Moreover, cheeses acidity was determined according to the American Association of Official Agricultural Chemists procedures (AOAC, 2005).

2.5 In vitro Antibacterial Effect of Bovine Lactoferrin against Foodborne Pathogens

Effect of LF on bacterial growth was examined using agar well diffusion test according to Motta and Brandelli (2002) to determine the appropriate concentration to be used in cheese. Briefly, TSA plates were prepared using semi-soft TSA (0.5%, w/v agar), 10 mL for each plate seeded with 100 µL (~5×10^6 CFU/mL) of overnight culture of one of the tested pathogens. An aliquot of 10-µL of each concentration of bovine lactoferrin was dotted on the surface of seeded lawns and kept to air dry for 5 min. The plates were incubated at 37°C and checked for zones of inhibition after 48 h.

2.6 Antibacterial Effect of Bovine Lactoferrin against Foodborne Pathogens inoculated in Karish cheese

Manufactured cheese curd was divided into equal parts and distributed in sterile containers. Cheese parts were inoculated with ~5×10^5 CFU/g of each tested pathogen. Then the containers were divided into two main groups as follow: *Control group (C),* cheese parts that were inoculated with *B. cereus, S. aureus, E. coli and L. monocytogenes*, without addition of LF. *Treated group (T),* cheese parts that were inoculated with the different foodborne pathogens and treated with bovine lactoferrin at 4% concentration.

Control and treated curds were repacked into polyethylene packages and stored at 5°C. 25 gm portions from each treatment were examined at zero time, 1st, 3rd, 5th and 7th days for determination of the counts of inoculated foodborne pathogen.

At each day of examination, 25 g cheese samples were homogenized with sodium citrate (2%) and tenfold serial dilutions were prepared, and samples were examined for the number of viable on Mannitol Yolk Polymyxin B agar (Oxoid), Baird-Parker agar (Oxoid) with egg yolk tellurite, Sorbitol MacConkey agar (Difco) and PALCAM Listeria Agar (Difco) media for *B. cereus, S. aureus, E. coli and L. monocytogenes* respectively, as described by APHA , 2004. The experiment was repeated 3 times and the average results for each treatment were recorded.

2.7 Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the statistical comparisons and the results were considered significantly different with $P < 0.05$ (Clark, 2009). The experiment was repeated in triplicates and average results for each group was tabulated.

3. RESULTS AND DISCUSSION

Lactoferrin is a protein that occurs naturally in milk and nowadays is increasingly supplemented in foods for its multiple functions and its application into food preservation is gaining great attention due to consumers' trend (Niaz et al., 2019). In this study the antibacterial effect of lactoferrin against *E. coli* O157:H7, *S. aureus L. monocytogenes*, and *B. cereus* inoculated in one of the most popular Egyptian soft cheese "Karish cheese" was evaluated.

The results of sensory evaluation revealed that the samples treated with the different concentrations of lactoferrin were generally acceptable, moreover the samples treated with higher concentrations of lactoferrin were given the highest score during the whole period of storage as shown in table 1.

The sensory evaluation of cheese with 4% lactoferrin indicated that addition of lactoferrin could improve the sensorial characteristics, appearance, odor and flavor, of Karish cheese.

The results for TA% determination showed slightly increase in TA% of samples treated with lactoferrin during the storage period, but the increase was lower than the control samples during the same storage period as shown in Table 2.

Antibacterial effect of 1%, 2% and 4% concentrations of bovine lactoferrin against foodborne pathogens were tested in vitro using agar well diffusion test to determine the appropriate concentration to be applied in cheese manufacturing. The results showed that the 4% lactoferrin concentration was the most inhibitory concentration against all the examined foodborne pathogens, *E. coli* O157:H7, *S. aureus L. monocytogenes*, and *B. cereus* on agar plates (Table 3).

Table 1. Sensorial evaluation (score 100 point) of the examined laboratory manufactured Karish cheese samples during refrigerated storage (5 ±1°C)

Examined sample*	0th day	5rd day	10th day	15th day	20th day
Appearance (20) (Mean± S.E)					
Control	17±0.03^{C**}	16±0.05^{B}	14±0.05^{D}	S	S
T1	18±0.01^{B}	16±0.05^{B}	15±0.07^{C}	13±0.05^{C}	S
T2	18±0.05^{B}	17±0.03^{AB}	16±0.01^{B}	14±0.05^{B}	14±0.05^{B}
T3	19±0.05^{A}	18±0.05^{A}	17±0.03^{A}	16±0.05^{A}	15±0.01^{A}
Body and texture (35) (Mean± S.E)					
Control	31±0.5^{C}	28±0.05^{D}	25±0.05^{D}	S	S
T1	31±0.28^{C}	29±0.02^{C}	26±0.05^{C}	22±0.05^{C}	S
T2	32 ±0.05^{B}	30±0.06^{B}	27±0.02^{B}	25±0.05^{B}	23±0.05^{B}
T3	34±0.05^{A}	32±0.05^{A}	29±0.01^{A}	27±0.05^{A}	25±0.02^{A}
Flavor (45) (Mean± S.E)					
Control	40±0.2^{C*}	35±0.01^{D}	28±0.05^{D}	S	S
T1	40±00.5^{C}	37±0.02^{C}	30±0.05^{C}	27±0.01^{C}	S
T2	41.5±0.5^{B}	39±0.03^{B}	37.5±0.05^{B}	34±0.1^{B}	29±0.05^{B}
T3	43±0.3^{A}	41±0.05^{A}	39±0.05^{A}	36±0.05^{A}	32±0.02^{A}

*****Control: cheese without bovine lactoferrin, T1: cheese with 1% bovine lactoferrin, T2: cheese with 2% bovine lactoferrin, T3: cheese with 4% bovine lactoferrin, S: spoiled sample.
******The different letters in the same columns, express a statistically difference ($P < 0.05$).

Table 2. Influence of addition of bovine lactoferrin on titratable acidity (T.A%) of laboratory manufactured Karish cheese

Storage time (Days)	**Titratable acidity % (Mean± S.E)**			
	Control*	**T1**	**T2**	**T3**
0th	1.12±0.03^{A**}	1.08±0.05^{B}	1.00±0.02^{B}	0.98±0.05^{C}
5th	1.28±0.03^{A}	1.25±0.02^{B}	1.15±0.04^{B}	1.10±0.04^{C}
10th	1.45±0.01^{C}	1.32±0.05^{AB}	1.25±0.02^{A}	1.18±0.02^{C}
15th	S	1.40±0.01^{A}	1.31±0.03^{B}	1.24±0.04^{C}
20th	S	S	1.40±0.03^{A}	1.30±0.02^{B}

*Control: cheese without bovine lactoferrin, T1: cheese with 1% bovine lactoferrin, T2: cheese with 2% bovine lactoferrin, T3: cheese with 4% bovine lactoferrin, S: spoiled sample.
**Values in the same row having different superscripts differ significantly ($P < 0.05$).

Table 3. In vitro antibacterial activity of bovine lactoferrin on foodborne pathogens

Bovine lactoferrin Concentration (%)	**Zone of Inhibition (Mean± S.E)**			
	S. aureus	*B. cereus*	*E. coli* O157	*L. monocytogenes*
1	13.5±0.74^{a*}	12.5±0.05^{b}	6.5±0.05^{c}	13 ± 1.06^{a}
2	17 ± 0.96^{ab}	16 ± 0.03^{b}	8 ± 0.79^{c}	18± 0.5^{a}
4	20 ±0.05^{b}	18 ±0.86^{c}	10 ± 0.65^{d}	23 ± 0. 6^{a}

*Values in the same row having different superscripts differ significantly ($P < 0.05$).

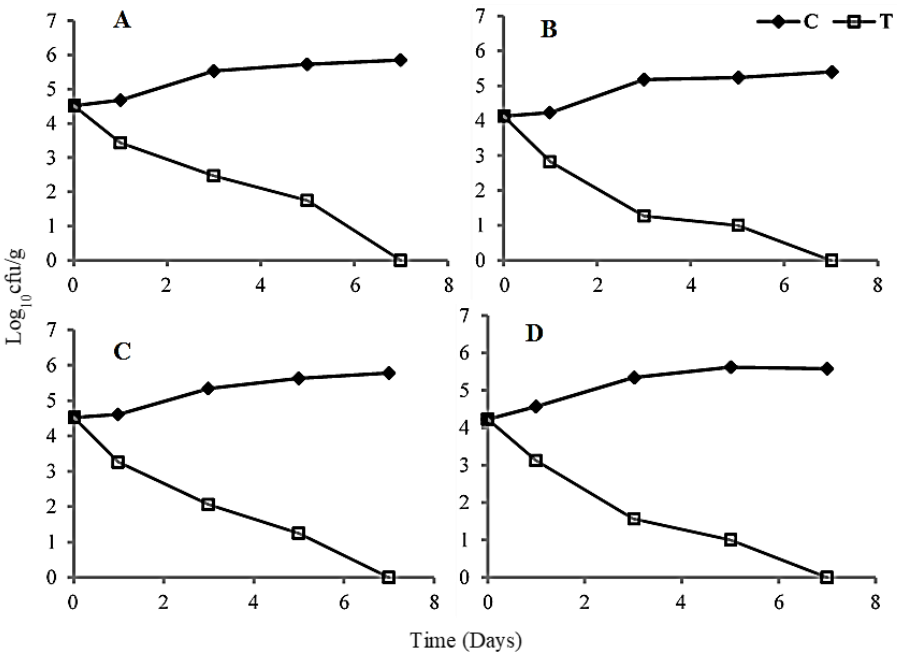

Figure 1. Antibacterial effect of bovine lactoferrin on (1) *E. coli* O157:H7, (2) *S. aureus,* (3) *B. cereus* and (4) *Listeria monocytogenes* inoculated in Karish cheese samples during their refrigerated storage.
***C:** refer to control which is Karish cheese inoculated with each pathogen without any lactoferin treatments; **T:** refer to Karish cheese inoculated with each pathogen and treated with 4% lactoferin.*

The 4% concentration was selected to investigate the antibacterial effect of lactoferrin against the before mentioned foodborne pathogens in Karish cheese. The results showed that this concentration of lactoferrin inhibited the examined pathogens and decreased their counts during the storage period (Figure 1). *S. aureus* and *L. monocytogenes* were almost totally inhibited at the 5th day of storage, while *E. coli* O157:H7 and *B. cereus* were totally inhibited at the 7th day of storage as shown in figure 1. In addition to inhibiting the tested pathogens, 4% lactoferrin extended the shelf life of Karish cheese (Table 1).

Several authors have reported antibacterial activity of lactoferrin in vitro against pathogens (Oliveira et al. 2001, Murdock and Matthews 2002, Murdock et al. 2007, Da Silva et al. 2012). In the same context Da Silva et al. (2012) reported that lactoferrin prevented the increase of *S. aureus* population in the cheeses. Murdock and Matthews (2002) concluded that, under conditions of low pH and refrigeration temperatures, lactoferrin hydrolysate can limit or reduce the populations of pathogenic bacteria as *E. coli* and *L. monocytogenes* in a dairy product

Lactoferrin antibacterial activity is attributed to its binding to iron, thus making it out-of-reach for the bacteria, and to the lipid component of lipopolysaccharide (LPS) and disruption of binding of other components to LPS in the bacterial cell that leads to interruption of the pathogenesis of enteropathogens by interfering with surface-expressed pathogenesis factors (Brandenburg et al., 2001, Ochoa and Cleary 2009).

CONCLUSION

Lactoferrin improved sensory properties of karish cheese experimentally inoculated with some food borne pathogens such as *E. coli* O157:H7, *S. aureus L. monocytogenes*, and *B. cereus*, in addition to extending its shelf life at cold storage. Therefore, the addition of lactoferrin to Karish could improve its microbiological safety.

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